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Effects of Glucocorticoids on the Beta-Adrenergic Adenylate Cyclase System of Pig Skin

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Effects of glucocorticoids on the epidermal beta-adrenergic adenylate cyclase system were investigated. Long-term incubation of pig skin slices in RPMI 1640 medium resulted in the gradual decrease in the epinephrine-induced cyclic AMP accumulations of skin. The addition of hydrocortisone (100 μM) in the incubation medium prevented this decrease, and after 24- and 48-h incubation, there was a marked difference in beta-adrenergic responsiveness between control and hydrocortisone-treated skin. The study using other steroid hormones revealed that this effect on the beta-adrenergic system was relatively specific for glucocorticoids. Hydrocortisone, prednisolone, dexamethasone, and betamethasone-17-valerate were shown to have marked effects on the beta-adrenergic system, while androstenedione, testosterone, dihydrotestosterone, progesterone, estrone, and beta-estradiol had no effect. Cortisone and estriol were shown to have similar but weaker effects than hydrocortisone. The effect of glucocorticoids was also relatively specific to the beta-adrenergic system, since there was no significant difference in adenosine- or histamine-induced cyclic AMP accumulations of skin after long-term incubation with and without hydrocortisone.

The mechanism of this glucocorticoid action does not seem to be through the simple protection of the beta-adrenergic system of the skin, since the addition of hydrocortisone in the incubation medium at 24 or 48 h incubation time, when the epinephrine-induced cyclic AMP accumulation was considerably decreased, reversed the epinephrine unresponsiveness of the skin, after the additional 24-h incubation. Furthermore, the effect of hydrocortisone was inhibited by 3 different kinds of inhibitors: (a) progesterone, an inhibitor of intracytoplasmic glucocorticoid receptor binding; (b) actinomycin D, an inhibitor of messenger RNA (mRNA) synthesis; and (c) cycloheximide, an inhibitor of protein synthesis at the translation step.

These results are in accordance with the view that glucocorticoids affect the beta-adrenergic system of epidermis by a mechanism requiring mRNA and protein synthesis possibly through the intracytoplasmic glucocorticoid receptor system of epidermis.

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The role of receptors of the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] system in mediating the effects of hormones and chemicals on target cells is well established [1,2]. With the exception of the soluble rat testicular enzyme [3], adenylate cyclase is a membrane-bound enzyme complex, and located at the outer cell membrane surface is the receptor component containing a specific site for binding of hormones and chemicals, which then initiates a train of events leading to the activation of adenylate cyclase (catalytic component) and to the formation of cyclic AMP. Previous data from our and other laboratories established that mammalian epidermis contains at least 4 independent receptor adenylate cyclase systems which respond respectively to catecholamines (beta-adrenergic agonists), to the prostaglandins (E_1 and E_2), to histamine (H_2 agonists), and to adenosine and its phosphorylated derivatives, resulting in the accumulation of cyclic AMP [4–6].

Among these 4 independent receptor adenylate cyclase systems in epidermis, the beta-adrenergic system has been characterized by its unstable or vulnerable nature, and epinephrine responsiveness of epidermis was shown to be easily lost by several in vivo or in vitro manipulations [7–9]. Defective beta-adrenergic responsiveness has also been noted in several pathologic conditions of epidermis [6,10].

Previously we have reported that the long-term incubation of pig skin (epidermal) slices resulted in a gradual decrease in beta-adrenergic responsiveness of the skin and furthermore that hydrocortisone had apparently a protective effect on this beta-adrenergic system without a significant effect on cyclic AMP phosphodiesterase activities [11]. This observation was followed by the finding that pretreatment of the skin with hydrocortisone magnified the inhibitory effect of epinephrine on epidermal outgrowth and mitosis [12], suggesting that hydrocortisone might reveal its physiologic and/or pharmacologic effect through the protective effect on the beta-adrenergic adenylate cyclase system of epidermis.

Using the long-term incubation system, our study was undertaken to clarify the nature and the mechanism of this hydrocortisone effect on the beta-adrenergic adenylate cyclase system of epidermis. Through this study we have confirmed the previous finding of hydrocortisone effect on the beta-adrenergic system, and, using various steroids, we established the specificity of this glucocorticoid effect. The evidence that this glucocorticoid effect is not through the simple protection of the beta-adrenergic system but seems to be manifested through the glucocorticoid receptor-induced active protein synthesis process was also presented.

MATERIALS AND METHODS

Domestic pigs weighing about 10 kg were anesthesized with nembutal (Dainippon, Osaka, Japan) i.p. (dose 30 mg/kg), as previously described [13]. Fifteen minutes after the anesthesia, skin slices were taken from the backs of pigs using a Castroviejo keratome (Storz Instrument Co. St. Louis, Missouri). Keratome shims were selected to give an average thickness of 0.2 mm and the skin slices thus obtained were histologically checked and were confirmed to be predominantly epidermis (~ 80%). The skin slice was cut into 5 × 5 mm squares, washed 3 times in RPMI 1640 medium, and floated with their keratin layers up in 10 ml of RPMI 1640 medium with added antibiotics (100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml fungizone) and various steroids to be tested. The incubations were done essentially according to the method of Flaxman and Harper [14] at 37°C in an atmosphere of 5% CO_2 in air. Since serum contains an unknown amount of growth factors which include glucocorticoids, serum was not added in the incubation medium. Under these conditions using chemically defined medium, epidermis was well maintained for up to at least 72 h [11,14]. Steroids were dissolved in ethanol and the final concentration of ethanol was 0.5% v/v. As control, only ethanol was added to the medium. As previously described [11], ethanol had no effect on the cyclic AMP levels of the skin at this concentration. After an appropriate time, the skin squares were transferred and floated in new RPMI 1640 medium at 37°C for cyclic AMP accumulation studies. Skin squares were preincubated at 37°C for 15 min to standardize the cyclic AMP level and after the preincubation 2 squares were randomly selected and floated in RPMI 1640 medium containing the various chemicals to be tested. The concentrations of epinephrine, histamine, and adenosine added for the cyclic AMP accumulation were 50 μM, 1 mM, and 2 mM, respectively. Previously it was shown that the concentrations of these chemicals were sufficient for the maximal accumulation of cyclic AMP, which was attained after 5 min without cyclic AMP phosphodiesterase inhibitors [15–17]. After the incubation at 37°C in a water bath (for 5 min unless otherwise stated), skin squares were quickly frozen between 2 plates of dry ice. The cyclic AMP content in these skin squares was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co. Tokyo, Japan) after partial purification by the method of Yoshikawa et al [18].

Protein concentration was measured by the method of Lowry et al [19]. Chemicals and drugs were all prepared fresh before each experiment and the pH of the medium was adjusted to 7. The statistical significance was determined by Student's *t*-test except that no statistical significance is indicated in any of the tables or figures.

RPMI 1640 medium was purchased from GIBCO (Grand Island, New York). Epinephrine was the product of Daiichi Pharmaceutical Co. (Tokyo, Japan). Penicillin-streptomycin-fungizone mixture was obtained from M.A. Bioproducts (Walkersville, Maryland). Progesterone was from Merck & Co. Inc. (Rahway, New Jersey), testosterone from Mann Research Labs Inc. (New York, New York), dihydrotestosterone from Teikoku Zoki Co. (Tokyo), and estrone from Nakarai Chemicals Ltd, (Kyoto, Japan). Betamethasone-17-valerate was a generous gift from Shionogi Pharmaceutical Co. (Osaka, Japan). Actinomycin D and cycloheximide were from P-L Biochemical Inc. (Milwaukee, Wisconsin) and Boehringer Mannheim Gmbh (FRG), respectively. All other chemicals were obtained from Sigma Chemical Co. (St. Louis).

RESULTS

Fig 1 shows the time course of the effect of hydrocortisone on the beta-adrenergic adenylate cyclase system of the skin. When pig skin squares were incubated without hydrocortisone, the epinephrine-induced cyclic AMP accumulations of skin gradually decreased. The addition of hydrocortisone in the incubation medium prevented the decrease or even increased the beta-adrenergic responsiveness, and after 24 and 48 h incubation time, there was a marked difference in epinephrine responsiveness between control and hydrocortisone-treated skin. In these experiments, usually at least 6 h incubation time was required for the detection of the difference and, during the

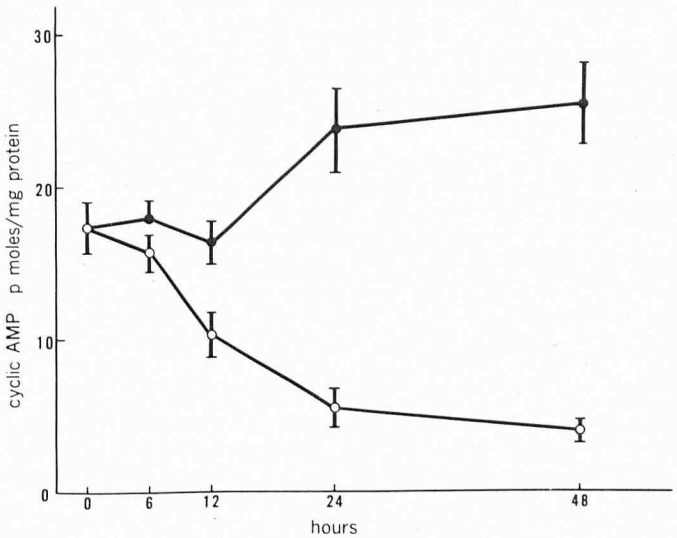

FIG 1. Effect of hydrocortisone on the beta-adrenergic adenylate cyclase system of pig epidermis. Skin squares were incubated in RPMI 1640 medium for an appropriate time with (●—●) and without (○—○) hydrocortisone. At the indicated time, epinephrine-stimulated cyclic AMP was measured after 5-min incubation with 50 μM epinephrine. Cyclic AMP level at 0 h indicates the epinephrine-stimulated cyclic AMP value of the skin without long-term incubation. Hydrocortisone was dissolved in ethanol and the final concentration of each chemical was 100 μM and 0.5% (v/v) respectively. Results are the means ± SE (n = 4).

first 3 h of incubation, there was no difference in epinephrine responsiveness between control and hydrocortisone-treated skin (data not shown). No histologic difference was noted between control and hydrocortisone-treated skin during the long-term incubation period and epidermal architecture was well maintained for up to 72 h (figure not shown).

That this effect of hydrocortisone is relatively specific to the beta-adrenergic system is shown in Fig 2. In contrast to the epinephrine responsivenesses, adenosine- and histamine-induced cyclic AMP accumulations of the skin gradually increased during the long-term incubation period. The addition of hydrocortisone in the incubation medium had no effect on adenosine- or histamine-induced cyclic AMP accumulations. Because of the weak response of the epidermal prostaglandin E adenylate cyclase system [5], the effect of hydrocortisone on this receptor system was not determined in the present study.

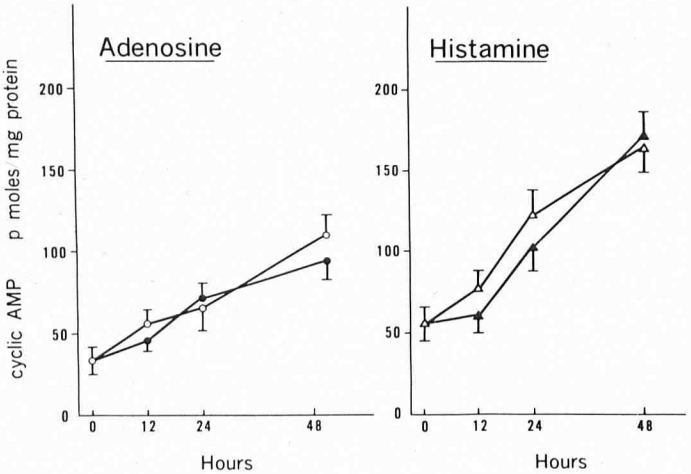

FIG 2. Effect of hydrocortisone on the adenosine and histamine (H_2) receptor adenylate cyclase systems of pig epidermis. Skin squares were incubated in RPMI 1640 medium exactly in the same way as in Fig 1. At the indicated time, adenosine- and histamine-stimulated cyclic AMP were measured after 5-min incubation with each chemical (adenosine, 2 mM; histamine, 1 mM). Results are the means ± SE (n = 4). ○—○ = Adenosine response of the control skin; ●—● = adenosine response of the hydrocortisone-treated skin; △—△ = histamine response of the control skin; ▲—▲ = histamine response of the hydrocortisone-treated skin.

TABLE I. *Steroid specificity in the modulation of beta-adrenergic adenylate cyclase responsiveness*

	Cyclic AMP (pmol/mg protein)	
	No addition	Epinephrine
Experimental series 1		
Control	0.7 ± 0.1	4.6 ± 0.6
Cortisone	0.9 ± 0.2	14.3 ± 1.2
Hydrocortisone	1.1 ± 0.2	22.4 ± 2.6
Prednisolone	1.1 ± 0.2	25.4 ± 4.6
Dexamethasone	0.9 ± 0.1	20.1 ± 2.2
Betamethasone-17-valerate	0.8 ± 0.1	23.3 ± 2.2
Experimental series 2		
Control	0.8 ± 0.1	4.9 ± 0.2
Androstenedione	0.8 ± 0.1	5.4 ± 0.9
Testosterone	0.9 ± 0.1	4.6 ± 0.4
Dihydrotestosterone	1.1 ± 0.1	6.8 ± 1.3
Hydrocortisone	1.1 ± 0.1	21.5 ± 1.2
Experimental series 3		
Control	1.2 ± 0.2	4.1 ± 0.5
Estrone	1.5 ± 0.2	4.4 ± 0.5
Estradiol	1.5 ± 0.3	6.9 ± 0.8
Estriol	1.4 ± 0.2	13.1 ± 1.9
Progesterone	0.7 ± 0.2	2.7 ± 0.2
Hydrocortisone	1.0 ± 0.2	30.5 ± 3.4

Skin squares were incubated in RPMI 1640 medium with various steroid hormones. After 48-h incubation, skin squares were incubated with 50 μM epinephrine for 5 min. No phosphodiesterase inhibitor was added in the media. Each steroid hormone was dissolved in ethanol and the final concentrations were 100 μM and 0.5%. Results are the means ± SE (n = 4).

TABLE II. *Effects of various inhibitors of glucocorticoid action*

	Cyclic AMP pmol/mg protein	
	No addition	Epinephrine
Experimental series 1		
Control	1.0 ± 0.1	5.2 ± 0.5
Progesterone (100 μM)	0.5 ± 0.1	2.5 ± 0.2
Hydrocortisone (100 μM)	0.8 ± 0.1	25.9 ± 7.5
Hydrocortisone (100 μM)		
+ progesterone (1 μM)	0.9 ± 0.2	28.6 ± 6.8
+ progesterone (10 μM)	1.1 ± 0.2	20.2 ± 5.2
+ progesterone (50 μM)	0.7 ± 0.1	12.2 ± 1.8
+ progesterone (100 μM)	0.5 ± 0.1	3.0 ± 0.4
Experimental series 2		
Control	1.6 ± 0.2	8.4 ± 1.7
Actinomycin D (5 μg/ml)	1.3 ± 0.3	7.0 ± 0.8
Hydrocortisone (100 μM)	1.7 ± 0.3	21.2 ± 3.8
Both agents	1.5 ± 0.2	10.2 ± 0.8
Experimental series 3		
Control	1.1 ± 0.2	4.5 ± 0.6
Cycloheximide (0.5 μM)	1.2 ± 0.3	5.2 ± 0.3
Hydrocortisone (100 μM)	1.1 ± 0.1	21.0 ±6.0
Both agents	1.2 ± 0.2	8.7 ± 0.4

Skin squares were incubated in RPMI 1640 medium with various chemicals. After 48-h incubation, skin squares were incubated with 50 μM epinephrine for 5 min. Results are the means ± SE (n = 4).

The effect of various steroids on the beta-adrenergic responsiveness of the skin is shown in Table I. Since the effect of hydrocortisone on beta-adrenergic responsiveness was considerably varied from experiment to experiment, the results of 3 typical experimental series are shown. In each series, the data of hydrocortisone-treated skin were included as a positive control in order to show a reliable estimation of the effect of each steroid. Glucocorticoids such as hydrocortisone, prednisolone, dexamethasone, and betamethasone-17-valerate were shown to have marked effects on the beta-adrenergic system of the skin and there was no difference in potency among the steroids at the concentration used (100 μM each). Androstenedione, testosterone, dihydrotestosterone, estrone, beta-estradiol, and progesterone had no effect on beta-adrenergic responsiveness. Cortisone and estriol were shown to have moderate effects.

The effects of progesterone, actinomycin D, and cycloheximide on the hydrocortisone effect are shown in Table II. Skin squares were incubated with hydrocortisone and/or various inhibitors, and epinephrine-induced cyclic AMP accumulations were investigated. When progesterone was added in the incubation medium, there was a marked inhibition of hydrocortisone effect. This inhibitory effect of progesterone was roughly dose dependent and at 100 μM concentration almost complete inhibition of hydrocortisone effect was observed. At 1 μM concentration, progesterone had no effect. Effect of hydrocortisone was also inhibited by the addition of 5 μg/ml actinomycin D, an inhibitor of transcription (mRNA synthesis) and by 0.5 μM cycloheximide, an inhibitor of protein synthesis at the translation step. There was no difference in epinephrine responsiveness between control and actinomycin D-treated skin (Table II, series 2) or cycloheximide-treated skin (Table II, series 3) at the concentrations used.

Fig 3 shows the reversal of epinephrine unresponsiveness by the addition of hydrocortisone. Hydrocortisone was added in the incubation medium at the indicated times (24 and 48 h), when the epinephrine responsiveness was considerably decreased (see also Fig 1). It was shown that the addition of hydrocortisone markedly increased the epinephrine responsiveness, and after each additional 24 h incubation time there was a marked difference in epinephrine responsiveness between control and hydrocortisone-treated skin. The epinephrine re-

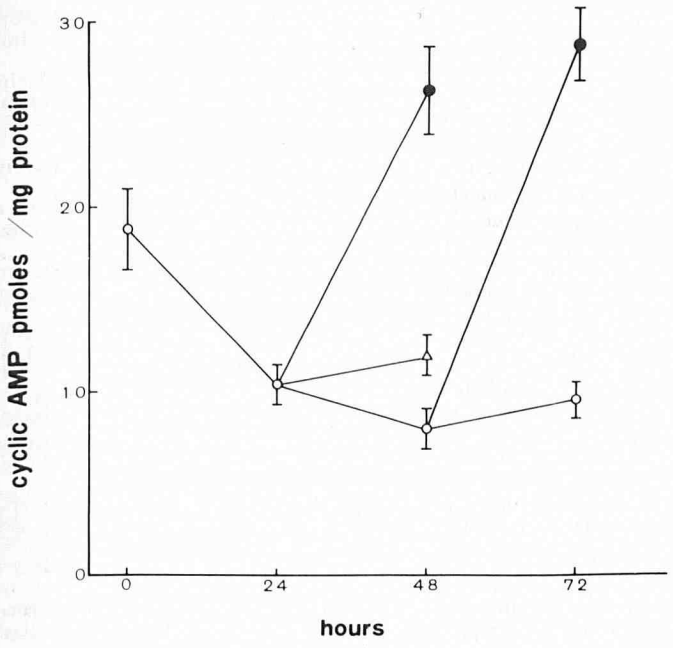

FIG 3. Reversal of epinephrine unresponsiveness by hydrocortisone. Skin squares were incubated in RPMI 1640 medium for the first 24 and 48 h without the addition of hydrocortisone. At the indicated time (24 and 48 h) skin squares were divided into groups and each group was incubated for the additional 24 h in a new RPMI 1640 medium with (●) and without (○) hydrocortisone. One group was incubated with 100 μM hydrocortisone plus 0.5 μM cycloheximide (△) at the 24-h incubation time. Epinephrine-stimulated cyclic AMP was measured at the indicated time as in Fig 1. Results are the means ± SE (n = 4).

sponse of the hydrocortisone-treated skin was shown to be more than the original response of the skin without the long-term incubation in some occasions as in this figure. This effect of hydrocortisone was also inhibited by the simultaneous addition of 0.5 μM cycloheximide (Fig 3, △).

DISCUSSION

Our data indicate that hydrocortisone relatively specifically works on the beta-adrenergic adenylate cyclase system in epidermis. Although there was a marked difference in epinephrine-induced cyclic AMP accumulations after long-term incubation with and without hydrocortisone (Fig 1), no difference in adenosine- or histamine-induced cyclic AMP accumulations was noted (Fig 2). Furthermore, in contrast to epinephrine responsiveness of the control skin, both adenosine and histamine responsivenesses gradually increased during the long-term incubation period (Fig 2). Previously we have reported that both low and high K_m cyclic AMP phosphodiesterase activities decreased during the long-term incubation period [11]. At 48 h incubation time, the low and high K_m phosphodiesterase activities were 78% and 66% of the original activities, respectively [11]. Since, in our system, incubations for cyclic AMP accumulations were carried out without the addition of phosphodiesterase inhibitors, the decreased phosphodiesterase activities might result in the apparent increase in adenosine- and histamine-induced cyclic AMP accumulation. Gradual decrease in epinephrine-induced cyclic AMP accumulations of the control skin despite the decreased phosphodiesterase activities, therefore, further strengthens the fact that the beta-adrenergic system is quite an unstable receptor system in epidermis.

The effect on the beta-adrenergic system was also specific to glucocorticoids. All the glucocorticoids, hydrocortisone, prednisolone, dexamethasone, and betamethasone-17-valerate were shown to have marked effects on the beta-adrenergic system of epidermis (Table I). Although there were no differences in potency among these steroids at the concentrations used (100 μM each), our recent observation suggests that potent glucocorticoids such as betamethasone-17-valerate reveal their effects with much lower concentrations than hydrocortisone [20]. Using the long-term incubation system, we are currently investigating the effect of various concentrations of steroids to establish the glucocorticoidal potency among them. Androgens (angrostenedione, testosterone, and dihydrotestosterone), estrogens (estrone and beta-estradiol), and progesterone had no effect on the beta-adrenergic system of epidermis. Cortisone and estriol were shown to have moderate effects (Table I). Cortisone, which in itself is a biologically inactive glucocorticoid [21], might be metabolized and reveal its effect as hydrocortisone, a finding observed in several other tissues [22,23].

There are several possibilities concerning the mechanism of this glucocorticoid effect. Lysosomal membrane stabilization has been proposed for some time as a mechanism for the anti-inflammatory effect of glucocorticoids [24,25]. Lysosomes contain digestive enzymes including proteases, nucleases, phosphatases, etc. Since previous data from our laboratory showed that the beta-adrenergic system was the most sensitive receptor system in epidermis to protease treatment [7], and since hydrocortisone apparently protected and preserved the beta-adrenergic responsiveness after a long-term incubation period, we formerly proposed this mechanism as the possible hydrocortisone effect [11]. Our present study, however, is contradictory to the simple protection mechanism. The effect of hydrocortisone was inhibited by protein synthesis inhibitors (Table II). The addition of hydrocortisone in the incubation medium at 24 and 48 h incubation time markedly increased the epinephrine responsivenesses of the skin almost to or more than the initial response of the skin (Fig 3). Although hydrocortisone stabilizes lysosomal membrane of the liver as was originally described by de Duve et al [24], recent studies using various other cell systems have failed to document the effect of hydrocortisone, and the stabilization hypothesis has been challenged [26].

Another possibility is that glucocorticoid reveals its effect through the classical steroid receptor pathway. According to the current view of the glucocorticoid action [27], in target cells, glucocorticoids bind to specific cytoplasmic receptor protein which becomes activated and translocates into nuclei. There, this hormone-receptor complex interacts with chromatin, leading to specific mRNA and consequent specific protein synthesis, eventually resulting in the observed phenotypic responses of target cells. In epidermis the existence of the glucocorticoid receptor system was established just recently [28–30] and it is quite likely that the effect of glucocorticoids on the beta-adrenergic system is through this steroid receptor system. Consistent with the time-consuming protein synthesis process, it required more than a 6-h lag period to detect the effect of glucocorticoid (Fig 1). The effect of glucocorticoid was inhibited by different kinds of inhibitors which work at 3 distinct sites of the glucocorticoid receptor pathway (Table II, Fig 3). Progesterone has been known to bind to intracytoplasmic glucocorticoid receptor and reveal its antiglucocorticoidal activity [31, 32]. Actinomycin D and cycloheximide are the classical inhibitors of protein synthesis at the transcription and translation step, respectively. Although not conclusive, these findings suggest that glucocorticoid reveals its effect through the specific protein synthesis process and this specific protein in turn seems to regulate the beta-adrenergic adenylate cyclase system of epidermis. Consistent with our finding, there are several other systems in which glucocorticoids induce the expression of various components of the beta-adrenergic adenylate cyclase system [33–36].

It should be noted that 100 μM hydrocortisone is a concentration far higher than is needed to saturate glucocorticoid receptors in vitro in cell-free systems [28–30]. Previously we reported that no effect was observed at 0.1 μM hydrocortisone [11], which is apparently more than enough for the saturation of epidermal glucocorticoid receptors in vitro [28]. Furthermore, the inhibitory effect of progesterone on the glucocorticoid effect (Table

II) was stronger than our expected value. Since progesterone binds less well to epidermal glucocorticoid receptors than hydrocortisone [29], it was surprising that half as much progesterone (50 μM) caused 50% inhibition of the 100 μM hydrocortisone effect (Table II). The reasons for these discrepancies remain unknown at present. Since we used an intact skin (epidermal) slice system, there would be other factors besides glucocorticoid receptor binding that would affect the expression of glucocorticoid effects, such as diffusion and metabolism of steroids. Thus our system might be relevant to in vivo findings; for example, that clinically useful glucocorticoids attain an epidermal concentration far greater than that needed to saturate glucocorticoid receptors [37].

As was discussed by Voorhees et al [38], it has been acknowledged that glucocorticoids and cyclic AMP induce similar changes in epidermis (see also [11]). However, it has been suggested that these 2 compounds work independently and there was scanty evidence on their interactions. Our findings clearly indicate that glucocorticoids work on the cyclic AMP system through regulation of the beta-adrenergic adenylate cyclase system, the most unstable receptor system in epidermis. Although this might not be the only effect of glucocorticoids on the epidermal cyclic AMP system, this effect of glucocorticoids might be significantly involved in physiologic and pharmacologic effects of these compounds on epidermis, which might be related to the therapeutic effect of steroids on the pathologic conditions of the skin such as psoriatic-involved epidermis where the defective beta-adrenergic responsiveness has been noted repeatedly [10,39,40].

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